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DOI:

[10.1016/j.etap.2013.01.007](https://doi.org/10.1016/j.etap.2013.01.007)

Document Version

Peer reviewed version

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Citation for published version (APA):

Nitzsche, D., Melzig, M. F., & Arlt, V. M. (2013). Evaluation of the cytotoxicity and genotoxicity of aristolochic acid I - A component of Aristolochiaceae plant extracts used in homeopathy. *ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY*, 35(2), 325-334. <https://doi.org/10.1016/j.etap.2013.01.007>

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The published version is available at:

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Manuscript

Evaluation of the cytotoxicity and genotoxicity of aristolochic acid I – a component of *Aristolochiaceae* plant extracts used in homeopathy

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Abstract

The medicinal plants *Aristolochia clematitis* L. as well as *Asarum europaeum* L., representatives of the plant family *Aristolochiaceae* and mentioned in the German Homeopathic Pharmacopoeia, contain aristolochic acid. We found that the mother tinctures of *A. clematitis* and *A. europaeum* inhibited DNA synthesis in human hepatoma HepG2 cells in a dose-dependent manner. One of the components of the plant extract, aristolochic acid I (AAI), is linked to the development of nephropathy and urothelial cancer in humans. Therefore, we also evaluated the cytotoxicity and genotoxicity of AAI in HepG2 cells. Cell proliferation was inhibited concentration-dependently by AAI using BrdU-ELISA and colony forming assay. AAI formed DNA adducts (measured by ^{32}P -postlabeling), induced chromosomal aberrations (micronuclei) and DNA strand breaks. DNA damage induced by AAI led to an arrest of cells in the *S*-phase which was associated with the increased expression of p53 and p21 proteins. The results are discussed under consideration of former studies.

Key words: Aristolochic acid I; HepG2 cells; DNA damage; *Aristolochia clematitis*; *Asarum europaeum*

Abbreviations: AAI, aristolochic acid I; HAB, German Homeopathic Pharmacopoeia; NQO1, NAD(P)H:quinone oxidoreductase

1. Introduction

In the German Homeopathic Pharmacopoeia (Homöopathisches Arzneibuch, HAB) the medicinal plants *Aristolochia clematitis* L. as well as *Asarum europaeum* L. are listed. The homeopathic tinctures of *A. clematitis* are used *e.g.* for the treatment of the upper and lower respiratory tract, the urinary and gastro-intestinal tract as well as at gynecological indications. The homeopathic tinctures of *A. europaeum* are applied for diseases of the central nervous system, lower respiratory tract, the gastrointestinal tract, and for flu-like infection (HAB, 2010). Many herbs from the genus *Aristolochia* and several species of the genus *Asarum*, both members of the plant family *Aristolochaceae*, contain aristolochic acid (AA) (Hashimoto et al., 1999). AA is a mixture of structurally related nitrophenanthrene carboxylic acids, mainly aristolochic acid I (AAI) (Pailer et al., 1956).

AA has been shown to be nephrotoxic and carcinogenic in rodents (reviewed in Arlt et al., 2002b). In humans AA is the cause of Chinese herb nephropathy, now termed aristolochic acid nephropathy (AAN), which is characterized by renal interstitial fibrosis and associated with a high risk of urothelial cancer (Debelle et al., 2008; Nortier et al., 2000). The cause of this disease has been tracked to herbal medicinal remedies containing *Aristolochia* herbs and due to these findings, *Aristolochia* spp. and herbs, that can be confused or substituted for *Aristolochia*, have been banned in many countries. AA has also been shown to play a role in the development of Balkan endemic nephropathy (BEN) which affects residents in certain rural areas of Romania, Croatia, Bosnia, Serbia and Bulgaria along the Danube river basin (Arlt et al., 2002a; Grollman et al., 2007; Jelakovic et al., 2012; Schmeiser et al., 2012). The contamination of grain by seeds of *A. clematitis*, which occurs widely in the wheat fields of the Balkan region, is discussed to be a cause of BEN (Arlt et al., 2007; Grollman et al., 2007).

AA was recently classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC) (Grosse et al., 2009).

The renal toxicity of AA and the development of urothelial carcinoma have been attributed primarily to AAI (Schmeiser et al., 2009; Shibutani et al., 2007). The genotoxic effect is based on the covalent binding of AAI to DNA after metabolic activation (Stiborova et al., 2008). In humans reductive bioactivation of AAI is attributed to NAD(P)H:quinone oxidoreductase (NQO1), cytochrome P450 (CYP) 1A1 and CYP1A2 (Stiborova et al., 2012; Stiborova et al., 2011). The proposed metabolic activation and DNA adduct formation of AAI is shown in Figure 1. AAI is activated by nitroreduction to *N*-hydroxyaristolactam I which can form an aristolactam-nitrenium ion as the ultimate carcinogen that binds to DNA, forming adducts. The structures of the major AAI-DNA adducts have been elucidated spectroscopically as 7-(deoxyadenosine-*N*⁶-yl)aristolactam I (dA-AAI) and 7-deoxyguanosine-*N*²-yl)aristolactam I (dG-AAI) (Schmeiser et al., 2009). Translesion DNA synthesis past dA-AAI adducts generates predominantly A to T transversions, which is AA's signature mutation (Arlt et al., 2007). It has been shown that characteristic A to T transversion mutations induced by AA lead to the activation of oncogenes (*i.e.* *H-ras*) in rodents or the inactivation of tumour suppressor genes (*i.e.* *TP53*) in humans (Chen et al., 2012; Grollman et al., 2007; Wang et al., 2012; Wang et al., 2011). Characteristic A to T transversion mutation induced by AA have also been observed in transgenic rodent mutations assays (Chen et al., 2006; Kohara et al., 2002; McDaniel et al., 2012; Mei et al., 2006; Xing et al., 2012) as well as in immortalised Hupki (human *TP53* knock-in) fibroblasts treated with AAI (Kucab et al., 2010; Nedelko et al., 2009).

The aim of the present study was to assess the cytotoxicity and genotoxicity of AAI in metabolically competent human hepatoma HepG2 cells (Westerink and Schoonen, 2007a, b). Assessment of genotoxicity was done by the ³²P-postlabeling assay, alkaline comet assay and

micronucleus assay. Furthermore, the impact of AAI treatment on cell cycle progression (measured by FACS analysis), and on NQO1, p53 and p21 protein levels (determined by Western blotting) was analyzed. In addition, we also measured cell viability and cell proliferation of HepG2 cells treated with AAI or the mother tinctures *A. clematitis* and *A. europaeum*.

2. Materials and methods

2.1. Chemicals

RPMI 1640 medium, fetal bovine serum (FBS), L-alanyl-L-glutamine, trypsin, phosphate-buffered saline (PBS) were supplied by Biochrom (Berlin, Germany). Aristolochic acid I (AAI; as free acid) was purchased from Sigma-Aldrich (Steinheim, Germany). Mother tinctures according to HAB 2010 of *Aristolochia clematitis* and *Asarum europaeum* were obtained from the German Homeopathic Union (Deutsche Homöopathie-Union, DHU, Karlsruhe, Germany). Unless indicated otherwise all chemicals were products of Sigma-Aldrich.

2.2. Cell culture

HepG2 cells were obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. HepG2 cells were cultivated in a humidified 5% CO₂ atmosphere at 37°C in RPMI 1640 supplemented with 10% FBS and 1% L-alanyl-L-glutamine. For experiments, cells were seeded into culture flasks or multi-well plates and adapted for 24 h. Stock solution of AAI (10 mg/mL) was prepared in DMSO. Controls were treated with DMSO only. In the experiments with the mother tinctures controls were handled with the corresponding ethanol / water mixture.

2.3. Cell proliferation assay

Cells were seeded in 96-well plates at a density of 2×10^4 cells per well. After 24 h of treatment with 0-20 µg/mL AAI and mother tinctures (up to 2.5% [v/v]), respectively, cells were labeled with BrdU for 2 h at 37°C. Cellular BrdU incorporation was measured using a cell proliferation ELISA BrdU colorimetric kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Absorbances at 450 nm, referenced at 620 nm, were measured

using a SPECTRA Fluor microplate reader (Tecan, Switzerland). To reduce unspecific binding of the antibody conjugate cells were incubated with 1% BSA (Roth, Karlsruhe, Germany) for 30 min before antibody incubation. Cell proliferation was calculated as percent of control.

2.4. Cell viability and colony forming ability

7.5×10^5 cells were transferred into T25 flasks and cultivated for 24 h. Subsequently, cells were treated with 0-20 $\mu\text{g/mL}$ AAI for 6 or 24 h, washed with PBS, trypsinized, collected in fresh medium and counted using a Casy Cell Counter (Schärfe Systems). 500 cells per culture dish (60 mm) were seeded in triplicate into fresh medium for the determination of colony forming ability. After 14 days of incubation, colonies were fixed with ethanol, stained with Giemsa (Roth, Karlsruhe, Germany), counted and calculated as percent of control.

2.5. DNA adduct analysis

2.25×10^6 cells were seeded into T75 flasks and after 24 h exposed to 0-20 $\mu\text{g/mL}$ AAI for 6 or 24 h. Subsequently, cells were harvested by trypsinization. Centrifugation at $400\times g$ and a washing step with PBS yielded a cell pellet, which was stored at -20°C until DNA isolation. DNA was isolated using a standard phenol/chloroform extraction method. DNA adducts were determined for each DNA sample (4 μg) using the nuclease P1 enrichment version of the ^{32}P -postlabeling method as described previously (Arlt et al., 2011; Phillips and Arlt, 2007). Chromatographic conditions for thin-layer chromatography (TLC) on polyethyleneimine-cellulose were: D1, 1.0 M sodium phosphate, pH 6.0; D2, 3.5 M lithium formate, 8.5 M urea, pH 4.0; and D3, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 9.0. TLC sheets were scanned by using a Packard Instant Imager (Dowers Grove, IL, USA). DNA

adduct levels (RAL, relative adduct labeling) were calculated from the adduct cpm, the specific activity of [γ - 32 P]ATP and the amount of DNA (pmol of DNA-P) used. Results were expressed as DNA adducts/ 10^8 nucleotides.

2.6. Single-cell gel electrophoresis (comet assay)

DNA strand breaks and formamidopyrimidine-DNA glycosylase (FPG)-sensitive sites were determined by the comet assay. The comet assay was performed essentially as described previously (Oya et al., 2011). Cells were seeded on six-well plates at a density of 3×10^5 cells per well. After treatment with 0-20 μ g/mL AAI for 24 h cells were trypsinized, centrifuged and resuspended in fresh medium. Microscope slides were pre-coated with 1.5% agarose at least one day in advance. A fraction of cell suspension was embedded in 0.65% low melting point agarose (37°C) and the mixture was spread over a slide. Slides were then placed in lysis buffer (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% Triton X-100) overnight at 4°C in the dark. After lysis treatment, slides were placed in enzyme buffer, followed by the addition of either 60 μ L enzyme buffer or 60 μ L FPG enzyme (54 ng/ μ L). FPG was a generous gift of A.R. Collins (University of Oslo, Norway). Incubations were performed in a humidity chamber (37°C, 30 min). DNA unwinding was performed in alkaline buffer (0.3 M NaOH, 1 mM EDTA) for 40 min at 4°C in the dark. Electrophoresis was performed (22 V, 24 min) using the same alkaline buffer. After electrophoresis, slides were neutralized, fixed in methanol and stained with ethidium bromide (10 μ g/mL). Cover slips were placed over the slides for computerized image analysis using Comet Assay IV software (Perceptive Instruments, Haverhill, UK). All slides were coded before analysis. Fifty nuclei were assessed per slide, and tail intensity (% tail DNA) was used as the measure of DNA damage induced, as recommended (Collins et al., 2008). The average tail intensity of two replicate treatments

was calculated. To calculate the FPG-sensitive sites % tail DNA of FPG-untreated samples was subtracted from % tail DNA of FPG-treated samples.

2.7. *In vitro micronucleus assay*

3.75×10^5 cells were transferred into T12.5 flasks. Subsequently, cells were treated with AAI in two parallel cultures for 6 h (0-50 $\mu\text{g/mL}$) or 24 h (0-20 $\mu\text{g/mL}$). After treatment cells were washed with PBS and incubated with medium containing cytochalasin B (final concentration 3 $\mu\text{g/mL}$). 72 h after transfer cells were collected by trypsinization. Aliquots were centrifuged directly onto microscope slides using Thermo Megafuge 1.0 (115 \times g for 5 min). Slides were fixed in 90% methanol for 10 min and stained with acridine orange (12.5 mg/100 mL in PBS; Invitrogen, Oregon, USA). Slides were scored using a fluorescence microscope (Olympus, Hamburg, Germany). All slides were coded before analysis. Micronuclei, as defined by criteria summarized by Fenech (2007), were scored in 1000 binucleated cells with two nuclei of approximately equal size. The replication index (RI) was calculated as $\text{RI} = (((\text{No. binucleated cells}) + (2 \times \text{No. multinucleate cells})) \div (\text{Total number of cells treated cultures}) / ((\text{No. binucleated cells}) + (2 \times \text{No. multinucleate cells})) \div (\text{Total number of cells control cultures})) \times 100$ (OECD, 2010).

2.8. *Flow cytometry*

For cell cycle analysis 7.5×10^5 cells were seeded into T25 flasks. After treatment with 0-20 $\mu\text{g/mL}$ AAI for 24 h cells were harvested by trypsinization. The cell pellet was resuspended in 0.2 mL PBS and fixed in 2 mL 70% ethanol (-20°C). Samples were stored at -20°C at least overnight. For flow cytometry analysis cells were spun down and resuspended in 2 mL staining buffer containing 40 $\mu\text{g/mL}$ propidium iodide (Invitrogen, UK) and 100 $\mu\text{g/mL}$ RNase A (Qiagen, Germany) in PBS. Cells were then incubated at 37°C for 1 h and

stored at 4°C overnight. Cell cycle analysis was performed using BD LSRII Flow Cytometer (BD Biosciences, UK). The relative number of cells in each phase of the cell cycle was determined using Cylchred and WinMDI v2.9 freeware (Hamouchene et al., 2011).

2.9. Western blot analysis

7.5×10^5 cells were seeded into T25 flasks. After treatment with 0-20 µg/mL AAI for 24 h cells were rinsed with cold PBS, lysis buffer (62.5 mM Tris, 1 mM EDTA, 10% glycerine, 2% SDS) was added, the lysate was scraped from the surface and stored at -20°C until Western blot analysis. Cell lysates were electrophoretically separated using NuPage 4-12% Bis-Tris SDS polyacrylamide gels (Invitrogen, UK). Following electrophoresis, gels were transferred onto a nitrocellulose membrane. The membrane was blocked in 4% non-fat dry milk (dissolved in Tris-buffered saline with 0.1% Tween). Blots were incubated with primary antibody and then with the species-specific horseradish peroxidase-conjugated secondary antibody (BioRad [anti-rabbit], Cell Signaling Technology [anti-mouse]). Bands were detected by chemiluminescence (West Pico Chemiluminescent Substrate, Thermo Scientific, USA). The monoclonal antibody against p53 (Ab-6) was purchased from Calbiochem (Darmstadt, Germany) and diluted 1:2000. Detection of p21 was done by monoclonal antibody purchased from BD Bioscience (Oxford, UK), diluted 1:2000. For detection of NQO1 a polyclonal antibody in a 1:20,000 dilution from Abcam (Cambridge, UK) was used. Monoclonal antibody to detect β-actin (AC-15) was also purchased from Abcam (Cambridge, UK), diluted 1:25,000 and used as loading control.

2.10. HPLC-UV analysis

Quantitative determination of AAI in the mother tincture was performed using high performance liquid chromatography (HPLC). HPLC-UV analysis was carried out on a

Hewlett Packard 1090 HPLC system equipped with a diode-array detector. 10 μ L of the mother tincture were injected onto a reversed-phase column (C-18 Eurospher-100, 250 mm x 4.6 mm, 5 μ m). An isocratic method (60:40; A:B; v/v), with a flow rate of 0.8 mL/min, was used. The HPLC mobile phase consisted of acetonitrile mixed with 0.1% acetic acid (A) and water (B). The detection wavelength was 250 nm.

2.11. Statistical analysis

The U-test was used for the statistical analysis of the data obtained in the cell proliferation assay, colony forming ability test, comet assay, micronucleus assay and cell cycle analysis. The significance was tested as $p < 0.05$ level. For the results of the DNA adduct analysis, signed rank test was used to analyze the differences from the solvent control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ was considered as statistically significant.

3. Results

3.1. Cell proliferation

The incorporation of BrdU during DNA synthesis in proliferating cells after incubation with 0-20 $\mu\text{g/mL}$ AAI was analyzed. After 24 h AAI showed a concentration-dependent inhibition of DNA synthesis in HepG2 cells (Fig. 2A). At a concentration of 20 $\mu\text{g/mL}$ AAI the DNA synthesis rate was decreased to about 25% relative to the solvent control. The proliferation assay was also used to determine the cytotoxicity of the mother tinctures of *A. clematidis* as well as *A. europaeum* containing AAI. After the addition of 1.25% [v/v] of the mother tincture of *A. clematidis* a significant inhibition of DNA synthesis was observed (Fig. 2B). Taking into account the content of 52 μg AAI per mL mother tincture determined by HPLC-UV, 1.25% [v/v] correspond to a concentration of 0.65 $\mu\text{g/mL}$ AAI. HepG2 cells treated with the mother tincture of *A. europaeum* also showed a significant decrease in proliferation. This was observed even at the lowest tested concentration of 0.25% [v/v] (Fig. 2C). Published by the Federal Institute for Drugs and Medical Devices (BfArM), the maximum amount of AAI in the mother tinctures of *A. europaeum* is 0.13 $\mu\text{g/g}$ (Thiele, 2010). Despite the lower content of AAI by a factor of 400 compared to *A. clematidis* tincture the mother tincture of *A. europaeum* was more cytotoxic in HepG2 cells. Both mother tinctures showed a higher cytotoxicity in comparison to the commercially available AAI, which may be due to other unknown components of the mother tinctures.

3.2. Cell viability and colony forming ability

Cell viability and colony forming ability were determined to examine the cytotoxic effects induced by AAI (Fig. 3). After treatment of HepG2 cells with up to 20 $\mu\text{g/mL}$ AAI for 6 or 24 h, no significant effect on cell viability (% control) was detectable. In contrast, there was a time- and concentration-dependent reduction of the colony forming ability. After 24 h

treatment with 10 µg/mL AAI the colony forming ability was reduced by approximately 50% and completely erased at 20 µg/mL AAI. A reduction of the colony forming ability was also observed at the shorter incubation period of 6 h; a reduction by 30% and 80% were seen at 10 and 20 µg/mL AAI, respectively. These results indicate that AAI induces long-lasting cytotoxic effect in cells.

3.3. Formation of DNA adducts

DNA adduct formation was determined using the nuclease P1 enrichment version of the ³²P-postlabeling method. HepG2 cells were treated with 0-20 µg/mL AAI for 6 or 24 h. The DNA adduct pattern induced by AAI on TLC consisted of two major adducts (Fig. 4A) which have been identified previously as 7-(deoxyadenosine-*N*⁶-yl)aristolactam I (dA-AAI) and 7-deoxyguanosine-*N*²-yl)aristolactam I (dG-AAI) (Arlt et al., 2011; Bieler et al., 1997). No DNA adducts were detectable in DNA from control cells treated with solvent (DMSO) only (data not shown). Quantitative ³²P-postlabeling analysis revealed a time- and concentration-dependent formation of AAI-DNA adducts (Fig. 4B). After 6 h the total AAI-DNA adduct level ranged from 7 to 229 adducts per 10⁸ nucleotides and after 24 h from 15 to 1043 adducts per 10⁸ nucleotides.

3.4. Induction of DNA strand breaks and FPG-sensitive sites

To assess the induction of DNA strand breaks/alkali-labile sites and formamidopyrimidine DNA glycosylase (FPG)-sensitive sites by AAI, HepG2 cells were treated with 0 or 20 µg/mL AAI for 24 h and DNA damage was measured as % tail DNA using the single-cell gel electrophoresis (comet) assay in combination with the bacterial repair enzyme FPG. Only low levels of DNA damage were detected after AAI exposure in the absence of FPG (Fig. 5); at 20 µg/mL AAI a statistically significant increase in % tail DNA

was observed compared to solvent control ($4.3 \pm 2.6\%$ versus $0.15 \pm 0.15\%$). Furthermore, an induction of FPG-sensitive sites by AAI was detected; at 20 $\mu\text{g/mL}$ AAI induced a 2.5-fold increase in % tail DNA relative to controls.

3.5. Analysis of micronuclei formation

HepG2 cells were treated with different concentrations of AAI for 6 or 24 h and micronuclei formation as well as cytotoxicity (replication index) was analyzed. A time- and concentration-dependent increase in micronuclei formation in binucleated cells was accompanied by increased cytotoxicity (Fig. 6). The formation of micronuclei was statistically significant at concentrations exceeding 30 $\mu\text{g/mL}$ AAI at 6 h and 15 $\mu\text{g/mL}$ at 24 h. Compared to background level, the micronuclei frequency increased more than 3-fold in cells treated with $\geq 15 \mu\text{g/mL}$ AAI for 24 h; the replication index was $49 \pm 3\%$ in cells treated with 15 $\mu\text{g/mL}$ AAI for 24 h. After 6 h the frequencies of micronucleated cells increased 2.2- and 4.0-fold after treatment with 30 and 50 $\mu\text{g/mL}$ AAI, respectively. The replication index after incubation with 30 $\mu\text{g/mL}$ AAI for 6 h was $60 \pm 12\%$. These observations are a strong indicator that AAI causes chromosome aberrations in HepG2 cells.

3.6. Cell cycle analysis

The DNA content of HepG2 cells exposed to 0-20 $\mu\text{g/mL}$ AAI for 24 h was measured by FACS analysis (Fig. 7). There was a clear accumulation of cells in the S phase of the cell cycle after exposure to 10 or 20 $\mu\text{g/mL}$ AAI, respectively. Arrest in the S phase implies that DNA synthesis is being inhibited in HepG2 cells in response to AAI exposure.

3.7. Western blot analysis

The impact of AAI treatment on the p53, p21 and NQO1 protein levels was investigated by Western blotting (Fig. 8). Protein levels were analyzed in HepG2 cells treated with 0-20 $\mu\text{g/mL}$ AAI for 24 h. P53 protein expression increased in a concentration-dependent manner after AAI treatment. Similarly, an accumulation of p21 was found. Furthermore, NQO1 was expressed but only slightly induced after treatment with 20 $\mu\text{g/mL}$ AAI.

4. Discussion

In the present study we evaluated the cytotoxic and genotoxic effect of AAI in human hepatoma HepG2 cells. Earlier studies determined specific AA-DNA adducts in rodents and human tissues by ^{32}P -postlabeling, especially the deoxyadenosine adduct 7-(deoxyadenosine- N^6 -yl)aristolactam I (dA-AAI). This DNA adduct is characterized by a high persistence in the target tissue and leads to the induction of AA-characteristic A to T transversion mutations in target tissue (reviewed in Arlt et al., 2002b; Arlt et al., 2007). In HepG2 cells we could also detect a time- and concentration-dependent formation of AAI-DNA adducts by ^{32}P -postlabeling. One of the two main adducts detected was 7-(deoxyadenosine- N^6 -yl)aristolactam I (dA-AAI). We showed that NQO1, which participates in AAI nitroreduction during metabolic activation (Stiborova et al., 2011), is expressed in HepG2 cells. We also found that AAI slightly induced NQO1 protein expression at the highest concentration. AAI may enhance its own metabolic activation through increased expression of NQO1.

Furthermore, AAI exposure resulted in an arrest of the HepG2 cells in *S*-phase of the cell cycle, indicating that interruption of DNA synthesis had occurred. Similar findings were observed previously in AAI-treated human colorectal carcinoma HCT116 cells (Simoes et al., 2008). A concentration-dependent inhibition of DNA synthesis in HepG2 cells has also been observed in the cell proliferation assay. The pause in DNA synthesis is probably due to the intra-*S* checkpoint, which allows repair enzymes to recognize damaged DNA and to repair it, avoiding irreversible errors during replication. The intra-*S*-phase checkpoint is usually activated by damages encountered during the *S*-phase or by unrepaired damages that escapes the *G1/S* checkpoint and leads to an interruption of replication (Sancar et al., 2004). Broschard et al. (1994) showed that AA-DNA adducts block DNA replication. Therefore, it seems likely that AA-DNA adducts did not activate the *G1/S* checkpoint in HepG2 cells. Generally, the *G1* arrest delays DNA damaged cells from progressing through the cell cycle,

avoiding accumulation of DNA damages by DNA repair or apoptosis (Sancar et al., 2004). Nevertheless, a concentration-dependent induction of p53 and p21 protein was demonstrated using Western blot analysis. Induction of p53 and p21 after AAI treatment has also been observed in previous studies (Arlt et al., 2011; Simoes et al., 2008). P53 and p21 proteins which are involved in the maintenance of the *G1/S* checkpoint also play a role in DNA repair mechanisms, so that the increased protein levels may be due to enhanced DNA repair.

In the comet assay only low levels of DNA strand breaks/alkali-labile sites were detected in HepG2 cells after AAI exposure in the absence of FPG. Wu et al. (2007) could also demonstrate the formation of DNA strand breaks/alkali-labile sites by AA in HepG2 cells. Furthermore, we found an induction of FPG-sensitive sites by AAI in HepG2 cells suggesting that AAI may also induce oxidative damage to DNA. The bacterial repair enzyme FPG recognizes 7,8-dihydro-8-oxoguanine (8-oxoguanine; 8-OH-dG), 2,6-diamino-4-hydroxy-5-formamindopyrimidine (Fapy-Gua), 4,6-diamino-5-formamido-pyrimidine (Fapy-Ade) and to a smaller extent 7,8-dihydro-8-oxoadenine (8-oxoadenine) as well as apurinic/apyrimidinic sites (AP sites) and converts them into DNA strand breaks by its associated DNA endonuclease activity (Boiteux et al., 1992; Tchou et al., 1991). Wu et al. (2007), who applied immunoperoxidative staining, showed 8-OH-dG formation in AA-exposed HepG2 cells. Further, Yu et al. (2011) showed that AAI induced oxidative stress-related DNA damage through the activation of the MEK/ERK1/2 signaling pathway and the depletion of intracellular glutathione. The authors concluded that DNA breakage after treatment with AAI is partially caused by the formation of radical oxygen species.

The ability of AAI to cause chromosome aberrations was analyzed in the micronucleus assay. A time- and concentration-dependent increase in micronuclei formation accompanied by enhanced cytotoxicity expressed as replication index in HepG2 cells was observed. This is in agreement with other studies which have shown the formation of micronuclei in response

to AA (Kevekordes et al., 2001; Wu et al., 2007). To examine the acute as well as long-term cytotoxicity of AAI cell viability and colony forming ability was determined. Up to the highest AAI concentration tested no effect was observed on cell viability. In contrast, there was a time- and concentration-dependent reduction of colony forming ability probably due to the inhibition of DNA replication by AA-DNA adducts. Results of the proliferation assay demonstrated that there was a concentration-dependent decrease in proliferation of HepG2 cells when treated with AAI. Also for the AAI-containing mother tinctures of *A. clematidis* and *A. europaeum* an inhibitory effect on cell proliferation was found which was, however, more pronounced. Comparing the inhibitory effect of the mother tinctures with the inhibition of DNA synthesis by the commercially available AAI it becomes clear that this effect is not attributable to AAI alone. Another well-known toxic substance found in the mother tincture *A. europaeum* is α -asarone (reviewed in Teuscher and Lindequist, 2010). In a recent study we showed that in the proliferation assay α -asarone exhibited a lower inhibition of the DNA synthesis than AAI at comparable concentrations (Unger and Melzig, 2012). The higher toxicity of the mother tinctures is probably due to other, not yet characterized toxic substances and/or synergistic interactions with other components.

There is increasing evidence that the plant extract AA and/or its major components (*i.e.* AAI) are responsible for the carcinogenic effects observed in humans who ingested *Aristolochia* plants or herbal medicines prepared from these plants (Schmeiser et al., 2009). Due to these findings, *Aristolochia* spp. and herbs that can be confused or substituted for *Aristolochia* have been banned in many countries. Unfortunately, these regulatory rules have been shown to be wholly inadequate in preventing exposure to AA (Gokmen and Lord, 2012; Gold and Slone, 2003), and there is growing evidence that AA exposure is causing a large unrecognized burden of disease in Asia with potentially devastating public health implications (Chen et al., 2012; Olivier et al., 2012). Therefore herbal medicines should be subjected to the

same stringent scrutiny and controls as drugs that undergo the standard development route before being launched on the market (Schmeiser et al., 2009). Increased efforts are made to assess the toxicity of herbal medicines (Ouedraogo et al., 2012) and our investigations provide a comprehensive example how to evaluate the impact of the plant component AAI on cell integrity. For this purpose we used the metabolically competent human hepatoma cell line HepG2 and analyzed the cytotoxic and genotoxic potential of AAI. Especially in the context for safe use of herbal remedies AA is a good example where improved regulation could help to eradicate a preventable disease – AAN.

Acknowledgments

The authors gratefully acknowledge Cornelia Görick for the HPLC analysis. Work at King's College London is supported by Cancer Research UK. Volker M. Arlt is a member of the European Union Network of Excellence ECNIS² (Environmental Cancer Risk, Nutrition and Individual Susceptibility).

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Legends to figures:

Figure 1: Metabolic activation and DNA adduct formation of AAI. 7-(deoxyadenosine- N^6 -yl)aristolactam I (dA-AAI), 7-deoxyguanosine- N^2 -yl)aristolactam I (dG-AAI).

Figure 2: Impact of (A) AAI as well as the mother tinctures of (B) *Aristolochia clematitis* and (C) *Asarum europaeum* on cell proliferation of HepG2 cells after 24 h exposure. Each bar represents the mean + SD of at least three independent experiments with six parallel samples. * $p < 0.05$; *** $p < 0.001$ Significantly different from the solvent control (U-test).

Figure 3: Impact of AAI on cell number and colony forming ability of HepG2 cells after exposure for 6 h and 24 h. Values represent the mean + SD of at least three independent experiments with three parallel samples. Cell numbers are not significantly different from the solvent control (Kruskal-Wallis). # $p < 0.05$; ** $p < 0.01$; ***, ### $p < 0.001$ Significantly different from the solvent control (U-test).

Figure 4: DNA adduct formation measured by ^{32}P -postlabeling in HepG2 cells after AAI exposure for 6 h and 24 h. (A) Autoradiographic profile of AAI-DNA adducts obtained after exposure to 20 $\mu\text{g/mL}$ AAI for 6 h and 24 h. (B) Each bar represents the mean of total AAI-DNA adducts (dA-AAI and dG-AAI) + SD of duplicate determinations of three independent experiments. AAI-DNA adducts were not detected in HepG2 control cells. * $p < 0.05$ Significantly different from the solvent control (signed rank test). dA-AAI: 7-(deoxyadenosin- N^6 -yl)aristolactam I; dG-AAI: 7-(deoxyguanosin- N^2 -yl)aristolactam I.

Figure 5: Induction of DNA strand breaks and FPG-sensitive sites in HepG2 cells exposed to AAI for 24 h. Each bar represents the mean + SD of duplicate determinations of two independent experiments with two parallel samples. *** $p < 0.001$ Significantly different from the solvent control (U-test). FPG: formamidopyrimidine-DNA glycosylase.

Figure 6: Induction of micronuclei in HepG2 cells exposed to AAI for (A) 6 h and (B) 24 h. Each bar represents the mean + SD of at least two independent experiments with two parallel samples. **, ^{##} $p < 0.01$; ***, ^{###} $p < 0.001$ Significantly different from the solvent control (U-test). MN: micronucleated cells; BNC: binucleated cells; RI: replication index.

Figure 7: Cell cycle analysis of HepG2 cells after exposure of AAI for 24 h using flow cytometry. Each bar represents the mean + SD of two independent experiments with three parallel samples. * $p < 0.05$; ** $p < 0.01$ Significantly different from the solvent control (U-test).

Figure 8: Western blot analysis of NQO1, p53 and p21 protein expression in HepG2 cells exposed to AAI for 24 h. β -actin protein expression was used as loading control.